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Title:

# SEARCHING SYSTEM FOR INSULIN-RESISTANT AMELIORANT WITHOUT POSSIBILITY OF EXACERBATING DIABETIC RETINOPATHY AND WITHOUT EDEMA INDUCING ACTION

Abstract:

PROBLEM TO BE SOLVED: To provide both a method for screening an insulin-resistant ameliorant without the possibility of exacerbating diabetic retinopathy and without edema inducing actions and the insulin-resistant ameliorant obtained by the method for screening. SOLUTION: This insulin-resistant ameliorant without the possibility of exacerbating the diabetic retinopathy and without the edema inducing actions is obtained by transducing a recombinant gene prepared by binding a human vascular endothelial growth factor gene containing a promoter region to a reporter gene into a mammalian cell and detecting the expression of the human vascular endothelial growth factor gene by the expression of the reporter gene of the cell.

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(71)出願人　593067000
岡　芳知
東京都世田谷区代沢１丁目13番９号
(72)発明者　岡　芳知
東京都世田谷区代沢１丁目13番９号
(74)代理人　100062144
弁理士　青山　葆　（外１名）

(54)【発明の名称】　糖尿病網膜症を増悪させる可能性がなく、浮腫惹起作用を有しないインスリン抵抗性改善薬の検索システム

(57)【要約】
【課題】糖尿病網膜症を増悪させる可能性がなく、浮腫惹起作用を有しないインスリン抵抗性改善薬のスクリーニング法、およびそれにより得られるインスリン抵抗性改善薬に関する。
【解決手段】プロモーター領域を含むヒト血管内皮増殖因子遺伝子にレポーター遺伝子を結合させた組換え遺伝子を哺乳動物細胞に導入し、この細胞のレポーター遺伝子発現によりヒト血管内皮増殖因子遺伝子の発現を検出することにより糖尿病網膜症を増悪させる可能性がなく、浮腫惹起作用を有しないインスリン抵抗性改善薬を得る。

【特許請求の範囲】
【請求項１】プロモーター領域を含むヒト血管内皮増殖因子遺伝子にレポーター遺伝子を結合させた組換え遺伝子を哺乳動物細胞に導入し、この細胞のレポーター遺伝子の発現によりヒト血管内皮増殖因子遺伝子の発現を検出することを特徴とする、糖尿病網膜症を増悪させる可能性がなく、浮腫惹起作用を有しないインスリン抵抗性改善薬のスクリーニング法。
【請求項２】レポーター遺伝子がルシフェラーゼ遺伝子である請求項１記載のスクリーニング法。
【請求項３】プロモーター領域を含むヒト血管内皮増殖因子遺伝子にレポーター遺伝子を結合させた組換え遺伝子。
【請求項４】インスリン抵抗性改善薬のスクリーニング法に用いるための請求項３記載の組換え遺伝子。
【請求項５】レポーター遺伝子がルシフェラーゼ遺伝子である請求項３記載の組換え遺伝子。
【請求項６】インスリン抵抗性改善薬のスクリーニング法に用いるための請求項５記載の組換え遺伝子。
【請求項７】請求項１または２に記載のスクリーニング法を用いることにより得られるインスリン抵抗性改善薬。
【請求項８】プロモーター領域を含むヒト血管内皮増殖因子遺伝子を、ベクターに組み込まれたレポーター遺伝子の上流にクローニングして得られる発現ベクターを哺乳動物細胞に導入し、この細胞のレポーター遺伝子の発現によりヒト血管内皮増殖因子遺伝子の発現を検出することを特徴とする、糖尿病網膜症を増悪させる可能性がなく、浮腫惹起作用を有しないインスリン抵抗性改善薬のスクリーニング法。
【請求項９】レポーター遺伝子がルシフェラーゼ遺伝子である請求項８記載のスクリーニング法。
【請求項１０】プロモーター領域を含むヒト血管内皮増殖因子遺伝子を、ベクターに組み込まれたレポーター遺伝子の上流にクローニングして得られる発現ベクター。
【請求項１１】インスリン抵抗性改善薬のスクリーニング法に用いるための請求項１０記載の発現ベクター。
【請求項１２】レポーター遺伝子がルシフェラーゼ遺伝子である請求項１０記載の発現ベクター。
【請求項１３】インスリン抵抗性改善薬のスクリーニング法に用いるための請求項１２記載の発現ベクター。
【請求項１４】請求項８または９に記載のスクリーニング法を用いることにより得られるインスリン抵抗性改善薬。
【発明の詳細な説明】
【０００１】糖尿病、特に２型糖尿病は、膵β細胞からのインスリン分泌低下とインスリン感受性の低下、すなわちインスリン抵抗性を特徴とする。生活習慣の欧米化に伴うインスリン抵抗性の増加は、現在、糖尿病が急増している最大の原因となっている。インスリン抵抗性改善剤には、日本ならびに海外で臨床使用されているピオグリタゾン（アクトス（登録商標））、海外で臨床使用されているロシグリタゾン（ＢＲＬ－４９６５３）、日本ならびに海外で臨床使用されていたが重篤な肝障害を生じる副作用のために２０００年３月に販売が停止されたトログリタゾン（ノスカール（登録商標））、ならびに開発中のＭＣＣ－５５５、、ＫＲＬ－４９６５３、およびＫＲＰ２９７などがあり、これらはいずれもチアゾリジン系の化合物である。また、非チアゾリジン骨格を有するものとしてＪＴＴ－５０１およびＹＭ４４０が開発中であり、その他に、ビグアナイド系薬剤と総称されるインスリン抵抗性改善剤として臨床使用されている塩酸メトフォルミン（グリコラン、メルビン（登録商標））、塩酸ブフォルミン（ジベトスＢ（登録商標））がある。
【０００２】すでに市販されているチアゾリジン誘導体系薬剤は浮腫（惹起作用）という副作用を有する。本発明者はこの副作用はチアゾリン誘導体が、それを投与した患者において、強力な血管透過性亢進因子でもある血管内皮増殖因子（vascular endothelial growth factor; ＶＥＧＦ）の血中濃度を増加させるためであること、さらに、これが血管内皮増殖因子をコードする遺伝子の発現を増加させるためであることを見いだした。また、ＶＥＧＦは糖尿病網膜症の発症・増悪の主要な因子であることが示されている（Aiello L.P.ら、N. Engl. J. Med. 331:1480-1487, 1994; Tanaka Y.ら、Lancet 349:1520, 1997; Adamis A.P.ら、Am. J. Ophthalmol. 118:445-450,1994; Miller J.W.ら、Am. J. Pathol. 145:574-584, 1994;およびTolentino M.J.ら、Ophthalmology 103:1820-1828, 1996参照）。
【０００３】チアゾリジン誘導体系薬剤が臨床使用されてまだ数年しか経っておらず、糖尿病網膜症の経過が比較的長いことから、該薬剤投与による糖尿病網膜症の悪化に関する報告はまだなされていないが、ＶＥＧＦが糖尿病網膜症を増悪させる主要な因子であり、その血中濃度がチアゾリジン誘導体系薬剤の投与により増加すること、さらに、糖尿病網膜症では症状の１つとして網膜に浮腫がみられることが知られており（後藤ら編、最新医学大事典、第２版（１９９６）、医歯薬出版、１２１３頁「糖尿病性網膜症」の項参照）、チアゾリジン誘導体系薬剤の副作用である浮腫自体も糖尿病網膜症悪化の一因となり得ることからも、チアゾリジン誘導体系薬剤投与による糖尿病網膜症の悪化は当然予期されることである。
【０００４】したがって、血中ＶＥＧＦレベルの増加を引き起こさないインスリン抵抗性改善剤は糖尿病網膜症を増悪させる可能性がなく、浮腫という副作用を生じない優れた薬剤となることが期待される。そこで、本発明者は、ＶＥＧＦ遺伝子発現を検出できる組換え遺伝子系を作製し、この組換え遺伝子系を導入した細胞を用いれ

ば、糖尿病網膜症を増悪させる可能性がなく、浮腫という副作用を生じない優れたインスリン抵抗性改善剤をスクリーニングすることができることを見いだし、本発明を完成した。

【０００５】すなわち、本発明は、プロモーター領域を含むヒト血管内皮増殖因子遺伝子にレポーター遺伝子を結合させた組換え遺伝子を哺乳動物細胞に導入し、この細胞のレポーター遺伝子発現によりヒト血管内皮増殖因子遺伝子の発現を検出することを特徴とする、糖尿病網膜症を増悪させる可能性がなく、浮腫惹起作用を有しないインスリン抵抗性改善薬のスクリーニング法を提供するものである。さらに本発明は、プロモーター領域を含むヒト血管内皮増殖因子遺伝子にレポーター遺伝子を結合させた組換え遺伝子を提供するものである。本発明の別の目的は、プロモーター領域を含むヒト血管内皮増殖因子遺伝子を、ベクターに組み込まれたレポーター遺伝子の上流にクローニングして得られる発現ベクターを哺乳動物細胞に導入し、この細胞のレポーター遺伝子の発現によりヒト血管内皮増殖因子遺伝子の発現を検出することを特徴とする、糖尿病網膜症を増悪させる可能性がなく、浮腫惹起作用を有しないインスリン抵抗性改善薬のスクリーニング法を提供することである。本発明のさらに別の目的は、プロモーター領域を含むヒト血管内皮増殖因子遺伝子を、ベクターに組み込まれたレポーター遺伝子の上流にクローニングして得られる発現ベクターを提供することである。本発明はまた、上記スクリーニング法を用いることにより得られるインスリン抵抗性改善薬をも提供するものである。本発明のスクリーニング法は、チアゾリジン系のみならずあらゆる種類のインスリン抵抗性改善薬の開発に広く応用可能であり、より安全なインスリン抵抗性改善薬を開発するためにはむしろぜひ応用すべきものである。本明細書において「レポーター遺伝子」は、本発明の目的に使用可能なあらゆるレポーター遺伝子を意味し、例えば、ルシフェラーゼ遺伝子およびクロラムフェニコールアセチルトランスフェラーゼ（ＣＡＴ）遺伝子などが含まれるがこれらに限定されるものではない。これらレポーター遺伝子は一般に市販されているものを使用することができる。好ましいレポーター遺伝子はルシフェラーゼ遺伝子である。以下の試験例および実施例において、本発明をさらに詳細に説明するが、これらは単に例示であって、本発明の範囲を何ら限定するものではない。

【０００６】試験例１

本発明者は、薬剤投与で認められる浮腫の臨床像が、血管透過性亢進でよく説明できることに着目し、チアゾリジン系薬剤であるトログリタゾン投与糖尿病患者において、強力な血管透過性亢進因子であるＶＥＧＦ（別名 Vascular permeability factor）の血清値を測定したところ、その平均値は１２０．１ｐｇ／ｍＬ（ｎ＝３０）であり、食事療法群（２９．２ｐｇ／ｍＬ、ｎ＝１０）、スルホニル尿素剤投与（ＳＵ）群（２５．８ｐｇ／ｍＬ、ｎ＝１０）、インスリン療法群（２４．６ｐｇ／ｍＬ、ｎ＝１０）と比較して有意（ｐ＜０．００１）に増加していた。５人の患者で投与前より経過を追ったところ、投与により上昇し、投与中止により前値に復した。さらに、３Ｔ３－Ｌ１脂肪細胞中のＶＥＧＦｍＲＮＡの発現は患者血中濃度に等しい濃度の、トログリタゾン添加および同様の作用機構を有するロシグリダゾン添加で増加した。

【０００７】実施例１

（１）ヒトVEGF遺伝子プロモーターの単離

既報のマウスVEGF mRNAの塩基配列（Claffey KPら、J. Biol. Chem. 267: 16317-16322, 1992; GenBank access on no. M95200）をもとにオリゴヌクレオチドプライマーを作成し、次いでRT-PCR法により3T3-L1脂肪細胞RNAよりマウスVEGFcDNA断片を得た。このcDNAをプローブとしてヒトゲノムDNAライブラリー（ストラタジーン社）をスクリーニングし、ヒトVEGF遺伝子（その上流約9 kbおよび下流約3 kbを含む）を単離した。

【０００８】（２）トログリタゾン応答領域を含むヒトVEGF遺伝子プロモーター／ルシフェラーゼレポータープラスミドの作成

上記（１）で単離したクローンから、ヒトVEGF遺伝子の転写開始点より上流約2.2 kbを含むDNA断片（-2274～+50, KpnI-NheI）を得た。このDNA断片をpGL3ベクター(プロメガ社)のルシフェラーゼ遺伝子の上流にクローニングし、VEGF遺伝子プロモーター／ルシフェラーゼを作成した。該レポータープラスミドphVEGF2.2LUCは、Escherichia coli JM109/phVEGF2.2LUCとして産業技術総合研究所生命工学工業技術研究所（茨城県つくば市東1丁目１番３号）に寄託された（受託番号：FERM P-18281、受託日：平成１３年３月３０日）。phVEGF2.2LUCをβガラクトシダーゼの発現プラスミドpCMV-β（クロンテック社）とともにA-172細胞（JCRB0228, JCRB Cell Bank）にリポフェクション（lipofection）法を用いて導入した。導入６～８時間後にトログリタゾン（20μM）添加、または非添加培養液に移し、さらに２４時間培養後、細胞を生理的食塩水で洗浄し、細胞溶解用緩衝液（プロメガ社）に溶解し、上清のルシフェラーゼ活性及びβガラクトシダーゼ活性を測定した。その結果、phVEGF2.2LUCを導入した細胞で、トログリタゾン添加によるルシフェラーゼ／βガラクトシダーゼ活性比の増加がみられ、phVEGF2.2LUCがトログリタゾン応答領域を含むVEGF遺伝子プロモーター／ルシフェラーゼレポータープラスミドであることが確認された。図１に得られたphVEGF2.2LUCの遺伝子マップを示す。

【０００９】（３）被検薬剤によるヒトVEGF遺伝子転写活性化のスクリーニング

上記（２）で得られたプラスミドが、広くインスリン抵抗性改善薬のVEGF遺伝子転写活性化能のスクリーニング

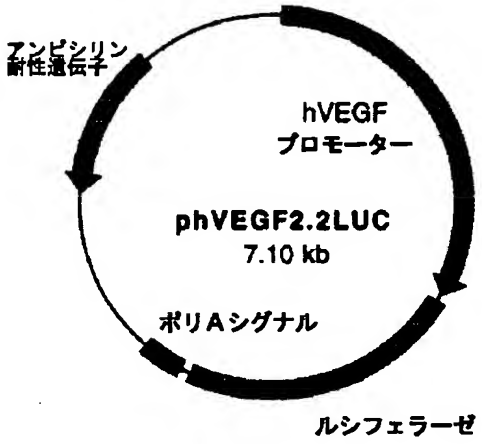
に利用できることを確認するため以下の実験を行なった。A-172細胞に上記（２）で作成したレポータープラスミドphVEGF2.2LUCおよびβガラクトシダーゼ発現プラスミドpCMV-βをリポフェクション法を用いて導入した（以降、本明細書ではここで得られた細胞をA-172VEGF細胞という）。レポータープラスミドおよびβガラクトシダーゼ発現プラスミド導入６～８時間後に被検薬剤（ピオクリタゾン20μM）またはトログリタゾン（20μM）を含む培養液に移し、さらに２４時間後に細胞を生理的食塩水で洗浄し、次いで細胞溶解用緩衝液に溶解した。細胞溶解液を遠心処理（12,000 g x 10秒）して得られた上清のルシフェラーゼ及びβガラクトシダーゼ活性を測定し、ルシフェラーゼ／βガラクトシダーゼ活性比を算出した。結果を図２に示す。図２に示した結果から明らかなように、トログリタゾンには強いVEGF遺伝子転写活性化能が見られ、一方、ピオグリタゾンのVEGF遺伝子転写活性化能はコントロールよりやや強いがトログリタゾンより弱かった。すなわち、この2剤を糖尿病網膜症を増悪させる可能性および浮腫惹起作用が低いという観点から比較すると、ピオグリタゾンのほうがトログリタゾンより優れていることがわかった。以上の結果から、本発明のスクリーニング法を用いて候補化合物をスクリーニングすることにより、VEGF遺伝子転写活性化能がより低い、さらには、患者に投与した際に糖尿病網膜症を増悪させる可能性がなく、浮腫惹起作用を有しないインスリン抵抗性改善薬を得ることができるだけでなく、既存のインスリン抵抗性改善薬の上記副作用を予測するのにも応用可能であることが明らかになった。

【図面の簡単な説明】

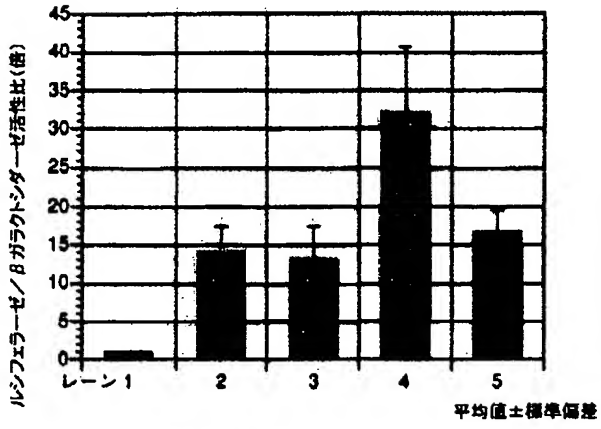
【図１】　トログリタゾン応答領域を含むVEGF遺伝子プロモーター／ルシフェラーゼレポータープラスミドphVEGF2.2LUCの遺伝子マップを示す。

【図２】　A-172VEGF細胞を用いた、チアゾリジン系インスリン抵抗性改善薬のVEGF遺伝子転写活性化能に関するスクリーニング実験の結果を示すグラフである。トログリタゾン存在下で培養したA-172VEGF細胞（レーン４）では、トログリタゾン非存在下で培養したA-172VEGF細胞（コントロール、レーン３）に比べて、その上清でのルシフェラーゼ／βガラクトシダーゼ活性比が著しく増大する。一方、ピオグリタゾン存在下で培養したA-172VEGF細胞（レーン５）ではルシフェラーゼ／βガラクトシダーゼ活性比の増大はコントロールよりやや高いが、トログリタゾン存在下より弱かった。pGL3（プロメガ社）だけを導入したA-172細胞（レーン１）およびCMVプロモーターをルシフェラーゼ遺伝子の上流に配置したpGL3-CMVを導入したA-172細胞（レーン２）の上清でのルシフェラーゼ／βガラクトシダーゼ活性比を、この細胞でのVEGFプロモーターの基礎活性を示すためのコントロールとして同時に示した。

【図１】




【図２】




レーン１：pGL3 導入細胞
レーン２：pGL3-CMV 導入細胞
レーン３：phVEGF2.2LUC 導入細胞
レーン４：phVEGF2.2LUC 導入細胞＋トログリタゾン 20μM
レーン５：phVEGF2.2LUC 導入細胞＋ピオグリタゾン 20μM

フロントページの続き

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| 33/68 | | C12N 15/00 | A | |

JP2001-340080
CLAIMS

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[Claim(s)]

[Claim 1] The screening procedure of insulin resistance improvement medicine which introduces into a mammalian cell the recombination gene which combined the reporter gene with the Homo sapiens blood vessel inner-bark growth factor gene including promoterregion, and is characterized by detecting Homo sapiens blood vessel inner-bark growth factor gene expression by the manifestation of the reporter gene of this cell and which a diabetic retinopathy must have been worsened and does not have an edema inducement operation.

[Claim 2] The screening procedure according to claim 1 whose reporter gene is a luciferase gene.

[Claim 3] The recombination gene which combined the reporter gene with the Homo sapiens blood vessel inner-bark growth factor gene including promoterregion.

[Claim 4] The recombination gene according to claim 3 for using for the screening procedure of insulin resistance improvement medicine.

[Claim 5] The recombination gene according to claim 3 whose reporter gene is a luciferase gene.

[Claim 6] The recombination gene according to claim 5 for using for the screening procedure of insulin resistance improvement medicine.

[Claim 7] Insulin resistance improvement medicine obtained by using a screening procedure according to claim 1 or 2.

[Claim 8] The screening procedure of insulin resistance improvement medicine which introduces into a mammalian cell the expression vector obtained by carrying out cloning of the Homo sapiens blood vessel inner-bark growth factor gene including promoterregion to the upstream of the reporter gene included in the vector, and is characterized by detecting Homo sapiens blood vessel inner-bark growth factor gene expression by the manifestation of the reporter gene of this cell and which a diabetic retinopathy must have been worsened and does not have an edema inducement operation.

[Claim 9] The screening procedure according to claim 8 whose reporter gene is a luciferase gene.

[Claim 10] The expression vector obtained by carrying out cloning of the Homo sapiens blood vessel inner-bark growth factor gene including promoterregion to the upstream of the reporter gene included in the vector.

[Claim 11] The expression vector according to claim 10 for using for the screening procedure of insulin resistance improvement medicine.

[Claim 12] The expression vector according to claim 10 whose reporter gene is a luciferase gene.

[Claim 13] The expression vector according to claim 12 for using for the screening procedure of insulin resistance improvement medicine.

[Claim 14] Insulin resistance improvement medicine obtained by using a screening procedure according to claim 8 or 9.

## DETAILED DESCRIPTION

[Detailed Description of the Invention]

[0001] Diabetes mellitus, especially type 2 diabetes are characterized by the insulin secretion fall from beta cells of pancreas and the fall of insulin susceptibility, i.e., insulin resistance. The increment in the insulin resistance accompanying West-izing of a lifestyle is the greatest cause by which current and diabetes mellitus are increasing rapidly. The pioglitazone by which clinical use is carried out Japan and overseas at the insulin resistance improvement agent (Actos (trademark)), ROSHIGURITAZON by which clinical use is carried out overseas (BRL-49653). The troglitazone by which sale was stopped in March, 2000 for the side effect which produces a critical hepatopathy although clinical use was carried out Japan and overseas (NOSUKARU (trademark)). And there are MCC-555 under development. KRL-49653, KRP297, etc., and each of these is the compounds of a thiazolidine system. Moreover, there are the hydrochloric-acid metformin (Glycoran, Melvin (trademark)) and hydrochloric-acid BUFORUMIN (Dibetos B (trademark)) by which clinical use is carried out as an insulin resistance improvement agent which JTT-501 and YM440 are developing as what has a non-thiazolidine frame, in addition is named BIGUANAIDO system drugs generically.

[0002] The already marketed thiazolidine derivative system drugs have a side effect of an edema (inducement operation). this invention person found out that this side effect is for making the blood drug concentration of the blood vessel inner-bark growth factor (vascular endothelial growth factor; VEGF) which is also a powerful permeability factor increase in the patient whom the thiazoline derivative medicated with it, and that it was for making the gene expression to which this carries out the code of the blood vessel inner-bark growth factor increase further. moreover, it is shown that VEGF(s) are the main factors of the onset and exacerbation of a diabetic retinopathy (Aiello L.P. et al. —) N.Engl.J.Med.331;1480-1487, 1994; Tanaka Y. et al., Lancet 349:1520, 1997; Adamis A.P. et al., Am.J.Ophthalmol.118:445- 450 and 1994; Miller J.W. et al. — Am.J.Pathol.145:574-584, 1994; and Tolentino M.J. et al., Ophthalmology 103:1820-1828, 1996 reference.

[0003] Clinical use of the thiazolidine derivative system drugs is carried out, and only several years still pass. Comparatively, although progress of a diabetic retinopathy is not made yet, a report of aggravation of the diabetic retinopathy by this medication from a ******** VEGF(s) are the main factors which worsen a diabetic retinopathy, and the blood drug concentration increases by administration of thiazolidine derivative system drugs, furthermore, in a diabetic retinopathy, it gets to know that an edema is seen by the retina as one of the symptoms — having — **** (edited by Goto —) Refer to the newest medicine important **, the 2nd edition (1996), Ishiyaku Publishers, and the term of 1213 pages "diabetic retinopathy". Aggravation of the diabetic retinopathy [ serve as / the edema itself which is the side effect of thiazolidine derivative system drugs / a cause of diabetic-retinopathy aggravation ] by thiazolidine derivative system medication is naturally expected.

[0004] Therefore, the insulin resistance improvement agent which does not cause the increment

in the VEGF level in blood cannot worsen a diabetic retinopathy, and becoming the outstanding drugs which do not produce the side effect of an edema is expected. Then, the recombination gene system which can detect VEGF gene expression was produced, and this invention person could not worsen a diabetic retinopathy, when using the cell which introduced this recombination gene system, he found out that the outstanding insulin resistance improvement agent which does not produce the side effect of an edema could be screened, and completed this invention.

[0005] That is, this invention offers the screening procedure of the insulin resistance improvement medicine which introduces into a mammalian cell the recombination gene which combined the reporter gene with the Homo sapiens blood vessel inner-bark growth factor gene including promoterregion, and is characterized by detecting Homo sapiens blood vessel inner-bark growth factor gene expression by the reporter gene manifestation of this cell and which a diabetic retinopathy must have been worsened and does not have an edema inducement operation. Furthermore, this invention offers the recombination gene which combined the reporter gene with the Homo sapiens blood vessel inner-bark growth factor gene including promoterregion. Another purpose of this invention is offering the screening procedure of insulin resistance improvement medicine which introduces into a mammalian cell the expression vector obtained by carrying out cloning of the Homo sapiens blood vessel inner-bark growth factor gene including promoterregion to the upstream of the reporter gene included in the vector, and is characterized by detecting Homo sapiens blood vessel inner-bark growth factor gene expression by the manifestation of the reporter gene of this cell and which a diabetic retinopathy's must have been worsened and does not have an edema inducement operation. Still more nearly another purpose of this invention is offering the expression vector obtained by carrying out cloning of the Homo sapiens blood vessel inner-bark growth factor gene including promoterregion to the upstream of the reporter gene included in the vector. This invention also offers the insulin resistance improvement medicine obtained by using the above-mentioned screening procedure again. The screening procedure of this invention is widely applicable to development of not only a thiazolidine system but all kinds of insulin resistance improvement medicine, and in order to develop a safer insulin resistance improvement medicine, it should surely be applied rather. Although a "reporter gene" means all usable reporter genes for the purpose of this invention in this specification, for example, a luciferase gene, a chloramphenicol acetyltransferase (CAT) gene, etc. are contained, it is not limited to these. These reporter genes can use what is generally marketed. A desirable reporter gene is a luciferase gene. In the following examples of a trial and examples, although this invention is further explained to a detail, these are only instantiation and do not limit the range of this invention at all.

[0006] In the troglitazone administration diabetic who is thiazolidine system drugs paying attention to blood-vessel-permeability sthenia being sufficient as the clinical feature of an edema accepted by medication, and the one example artificer of a trial being able to explain VEGF which is a powerful permeability factor (alias name Vascular permeability factor) The place which measured the blood serum value, The average is 120.1 pg/mL (n= 30), and is an alimentary therapy group (29.2 pg/mL). n= 10, a sulfonyl urea agent administration (SU) group (25.8 pg/mL,

n= 10), and an insulin therapy group (24.6 pg/mL, n= 10) — comparing — being significant (p< 0.001) — it was increasing. When progress was followed from administration before by five patients, it went up by administration and was restored to the last value by administration termination. Furthermore, the manifestation of VEGFmRNA in 3 T3-L1 fat cell increased by the ROSHIGURIDAZON addition which has troglitazone addition and the same mechanism of action of concentration equal to patient blood drug concentration.

[0007] The oligonucleotide primer was created based on the base sequence (Claffey KP et al., J.Biol.Chem.267:16317-16322, 1992; GenBank accesson no.M95200) of the mouse VEGF mRNA of a isolation previous report of an example 1(1) Homo-sapiens VEGF gene promotor, and, subsequently the mouse VEGFcDNA fragment was obtained from the 3 T3-L1 fat cell RNA by RT-PCR method. The human genome DNA library (Stratagene) was screened by having used this cDNA as the probe, and the Homo sapiens VEGF gene (that about 9 upstream kb and about 3 lower stream of a river kb are included) was isolated.

[0008] (2) The DNA fragment (-2274-+50, KpnI-NheI) which includes the about 2.2 upstream kb from the transcription initiation site of a Homo sapiens VEGF gene was obtained from the clone isolated by the creation above (1) of a Homo sapiens VEGF gene promotor / luciferase reporter plasmid including a troglitazone response field. Cloning of this DNA fragment was carried out to the upstream of the luciferase gene of pGL3 vector (pro megger company), and a VEGF gene promotor / luciferase was created. this — reporter plasmid phVEGF2.2LUC was deposited with National Institute of Bioscience and Human-Technology, the National Institute of Advanced Industrial Science and Technology, (1-1-3, Higashi, Tsukuba-shi, Ibaraki-ken) as Escherichia coli JM109/phVEGF2.2LUC (trust number: FERM P- 18281, a trust day : March 30, Heisei 13). phVEGF2.2LUC — manifestation plasmid pCMV-beta (Clontech) of a beta galactosidase — A-172 cell (JCRB0228, JCRB Cell Bank) — a RIPOFE cushion (lipofection) — it introduced using law. It moved to troglitazone (20microM) addition or non-adding culture medium 6 - 8 hours after installation, physiological sodium chloride solution washed the cell after 24 more hour culture, it dissolved in the buffer solution for lysis (pro megger company), and the luciferase activity and beta galactosidase activity of supernatant liquid were measured. Consequently, it was checked that they are the VEGF gene promotor / luciferase reporter plasmid in which the increment in the luciferase / beta galactosidase activity ratio by troglitazone addition is seen, and phVEGF2.2LUC includes a troglitazone response field in the cell which introduced phVEGF2.2LUC. The gene map of phVEGF2.2LUC obtained by drawing 1 is shown.

[0009] (3) The following experiments were conducted in order that the plasmid obtained by the screening above (2) of the Homo sapiens VEGF gene imprint activation by the tested drug agent might check that it can use for screening of the VEGF gene imprint activation ability of insulin resistance improvement medicine widely. Reporter plasmid phVEGF2.2LUC and beta galactosidase manifestation plasmid pCMV-beta which were created above (2) were introduced into A-172 cell using the RIPOFE cushion method (on these specifications, the cell obtained here is henceforth called A-172VEGF cell). It moved to the culture medium which contains a tested drug agent (PIOKURITAZON20microM) or troglitazone (20microM) a reporter plasmid and 6 - 8

hours after beta galactosidase manifestation plasmid installation, physiological sodium chloride solution washed the cell 24 more hours after, and, subsequently to the buffer solution for lysis, it dissolved. The luciferase and beta galactosidase activity of supernatant liquid which were acquired by carrying out centrifugal processing (12,000 g x 10 seconds) of the cell solution were measured, and luciferase / beta galactosidase activity ratio was computed. A result is shown in drawing 2 . VEGF gene imprint activation ability strong against troglitazone was seen, and although the VEGF gene imprint activation ability of pioglitazone was a little stronger than control, on the other hand, it was weaker than troglitazone, so that clearly from the result shown in drawing 2 . That is, when this 2 agent was compared from a viewpoint that possibility of worsening a diabetic retinopathy, and an edema inducement operation are low, it turned out that pioglitazone is superior to troglitazone. It became clear for it to be able to apply to it not only to be able to obtaining the insulin resistance improvement medicine with lower VEGF gene imprint activation ability which a diabetic retinopathy must have been further worsened when a patient is medicated, and does not have an edema inducement operation; but predicting the above-mentioned side effect of the existing insulin resistance improvement medicine by screening a candidate compound using the screening procedure of this invention from the above result.

## OPERATION

It has a side effect of (an inducement operation). this invention person found out that this side effect is for making the blood drug concentration of the blood vessel inner-bark growth factor (vascular endothelial growth factor; VEGF) which is also a powerful permeability factor increase in the patient whom the thiazoline derivative medicated with it, and that it was for making the gene expression to which this carries out the code of the blood vessel inner-bark growth factor increase further. moreover, it is shown that VEGF(s) are the main factors of the onset and exacerbation of a diabetic retinopathy (Aiello L.P. et al. —) N.Engl.J.Med.331:1480-1487, 1994; Tanaka Y. et al., Lancet 349:1520, 1997; Adamis A.P. et al., Am.J.Ophthalmol.118:445- 450 and 1994; Miller J.W. et al. — Am.J.Pathol.145:574-584, 1994; and Tolentino M.J. et al., Ophthalmology 103;1820-1828, 1996 reference.

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derivative system drugs / a cause of diabetic-retinopathy aggravation ] by thiazolidine derivative system medication is naturally expected.

[0004] Therefore, the insulin resistance improvement agent which does not cause the increment in the VEGF level in blood cannot worsen a diabetic retinopathy, and becoming the outstanding drugs which do not produce the side effect of an edema is expected. Then, the recombination gene system which can detect VEGF gene expression was produced, and this invention person could not worsen a diabetic retinopathy, when using the cell which introduced this recombination gene system, he found out that the outstanding insulin resistance improvement agent which does not produce the side effect of an edema could be screened, and completed this invention.

[0005] That is, this invention offers the screening procedure of the insulin resistance improvement medicine which introduces into a mammalian cell the recombination gene which combined the reporter gene with the Homo sapiens blood vessel inner-bark growth factor gene including promoterregion, and is characterized by detecting Homo sapiens blood vessel inner-bark growth factor gene expression by the reporter gene manifestation of this cell and which a diabetic retinopathy must have been worsened and does not have an edema inducement operation. Furthermore, this invention offers the recombination gene which combined the reporter gene with the Homo sapiens blood vessel inner-bark growth factor gene including promoterregion. Another purpose of this invention is offering the screening procedure of insulin resistance improvement medicine which introduces into a mammalian cell the expression vector obtained by carrying out cloning of the Homo sapiens blood vessel inner-bark growth factor gene including promoterregion to the upstream of the reporter gene included in the vector, and is characterized by detecting Homo sapiens blood vessel inner-bark growth factor gene expression by the manifestation of the reporter gene of this cell and which a diabetic retinopathy's must have been worsened and does not have an edema inducement operation. Still more nearly another purpose of this invention is offering the expression vector obtained by carrying out cloning of the Homo sapiens blood vessel inner-bark growth factor gene including promoterregion to the upstream of the reporter gene included in the vector. This invention also offers the insulin resistance improvement medicine obtained by using the above-mentioned screening procedure again. The screening procedure of this invention is widely applicable to development of not only a thiazolidine system but all kinds of insulin resistance improvement medicine, and in order to develop a safer insulin resistance improvement medicine, it should surely be applied rather. Although a "reporter gene" means all usable reporter genes for the purpose of this invention in this specification, for example, a luciferase gene, a chloramphenicol acetyltransferase (CAT) gene, etc. are contained, it is not limited to these. These reporter genes can use what is generally marketed. A desirable reporter gene is a luciferase gene. In the following examples of a trial and examples, although this invention is further explained to a detail, these are only instantiation and do not limit the range of this invention at all.

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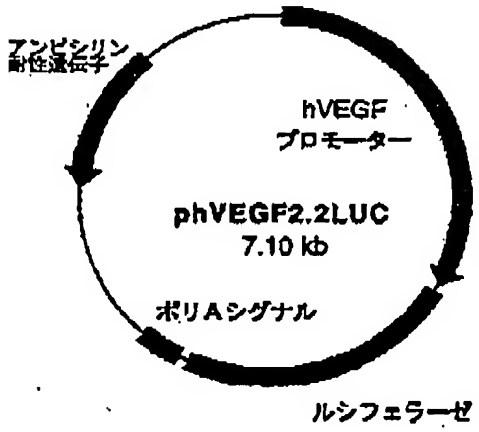
## DESCRIPTION OF DRAWINGS

[Brief Description of the Drawings]

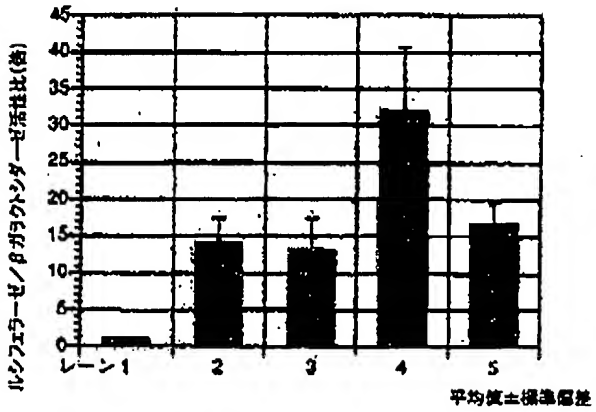
[Drawing 1] The gene map of a VEGF gene promotor / luciferase reporter plasmid phVEGF2.2LUC including a troglitazone response field is shown.

[Drawing 2] It is the graph using an A-172VEGF cell which shows the result of the screening experiment about the VEGF gene imprint activation ability of thiazolidine system insulin resistance improvement medicine. In the A-172VEGF cell (lane 4) cultivated under troglitazone existence, the luciferase / beta galactosidase activity ratio in the supernatant liquid increase remarkably compared with the A-172VEGF cell (control, lane 3) cultivated under troglitazone nonexistence. Although increase of luciferase / beta galactosidase activity ratio was a little higher than control in the A-172VEGF cell (lane 5) cultivated under pioglitazone existence on the other hand, it was weaker than the bottom of troglitazone existence. The luciferase / beta galactosidase activity ratio in the supernatant liquid of A-172 cell (lane 2) which introduced pGL3-CMV which has stationed A-172 cell (lane 1) and CMV promotor who introduced only pGL3 (pro megger company) for the upstream of a luciferase gene were shown in coincidence as control to show the basic activity of the VEGF promotor in this cell.

[Drawing 1]




[Drawing 2]




レーン1：pGL3 導入細胞
レーン2：pGL3-CMV 導入細胞
レーン3：phVEGP2.2LUC 導入細胞
レーン4：phVEGP2.2LUC 導入細胞＋トログリタゾン 20μM
レーン5：phVEGP2.2LUC 導入細胞＋ピオグリタゾン 20μM